(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 194 586 B1

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent:
12.01.2005 Bulletin 2005/02

(21) Application number: **00935235.2**

(22) Date of filing: **07.06.2000**

(51) Int Cl.[7]: **C12Q 1/02**, C12Q 1/22, C12Q 1/00

(86) International application number:
PCT/FI2000/000507

(87) International publication number:
WO 2000/075367 (14.12.2000 Gazette 2000/50)

(54) **A METHOD TO ENABLE ASSESSMENT OF GROWTH AND DEATH OF MICRO-ORGANISMS**

VERFAHREN UM WACHSTUM UND TOD VON MIKROORGANISMEN ZU BESTIMMEN

PROCEDE POUR REALISER UNE ANALYSE DE LA PROLIFERATION ET DE LA MORT DE MICRO-ORGANISMES

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE

(30) Priority: **07.06.1999 FI 991296**

(43) Date of publication of application:
10.04.2002 Bulletin 2002/15

(73) Proprietors:
- **Lilius, Esa-Matti**
  20780 Kaarina (FI)
- **Virta, Marko**
  20100 Turku (FI)

(72) Inventors:
- **Lilius, Esa-Matti**
  20780 Kaarina (FI)
- **Virta, Marko**
  20100 Turku (FI)

(74) Representative: **Heikkilä, Hannes Antero**
Turun Patenttitoimisto Oy,
P.O. Box 99
20521 Turku (FI)

(56) References cited:
WO-A1-98/14605
US-A- 5 164 301
US-A- 5 824 468

- **DATABASE WPI Week 199932, Derwent Publications Ltd., London, GB; AN 1999-379000, XP002909842 ZH TETSUDO SOGO GIJUTSU KENKYUSHO: 'Rapid identification of microorganism cell for measuring sterilization effect etc - involves using fluorescent pigments wich differ in their wavelengths for identifying dead and living cells' & JP 11 146 798 A 02 June 1999**
- **DATABASE BIOSIS [Online] FRATAMICO PINA M. ET AL.: 'Construction and characterization of escherichia coli 0157:H7 strains expressing firefly luciferase and green fluorescent protein and their use in survival studies', XP002909867 Retrieved from Dialog Information Services, accession no. 11223430 Database accession no. 199800004762**

Note: Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

EP 1 194 586 B1

Description

[0001] This invention relates to a method to enable the assessment of growth and death of a micro-organism within a chosen time period in an environment of interest.

BACKGROUND OF THE INVENTION

[0002] When studying growth and death of a micro-organism under the influence of specific environments, e.g. production and storage environments that e.g. could or could not be refrigerated, or involving chemicals or matrixes, e.g. antibiotics, microbial toxins, heavy metals and serum complement, microbial cultures are most often incubated for hours or days. In these circumstances death and growth occur simultaneously. If additionally some of the cells lyse, e.g. when analysing the serum complement, it is difficult to know to what one should compare the amount of living cells at the end of the experiment. Convenient methods to determine the number of living cells, e.g. by measuring luciferase bioluminescence, are known but if no more information is available it is impossible to assess to what extent growth or/and death of the micro-organisms takes or has taken place.

[0003] Growth rates and death rates of micro-organisms in specific environments are of interest in many areas. Death rates and growth rates of micro-organisms and especially harmful and/or pathogenic micro-organisms are of importance in risk assessments of products of the pharmaceutical industry and products for human consumption with regard to their production, storage and distribution to the consumers. Knowledge of death and growth rates of micro-organisms are of particular importance in specific applications such as in the development of antibiotics, disinfectants and bactericidal products or monitoring of sterilisation, disinfection and cleaning processes.

[0004] Reporter genes coding for luminescent or/and fluorescent products have been introduced to micro-organisms to enable the assessment of the quantity or survival of living micro-organisms (WO 96/23898, WO 98/14605, WO 98/30715, WO 98/36081, US 5,824,468). Even simultaneous use of luminescent and fluorescent markers has been used (Fratamico et al., Journal of Food Protection, Vol 50 No 10, 1997, 1167-1173). Luminescent and fluorescent markers have, however, only been used as markers for survival of micro-organisms and the use of two different markers within one micro-organism enabling the differentiation between growth and death rates has not been reported.

OBJECT AND SUMMARY OF THE INVENTION

[0005] The object of the present invention is to provide a method to enable the assessment of the growth and death of a micro-organism within a chosen time period in an environment of interest by introducing into said micro-organism a green fluorescent protein (GFP) and a luciferase reporter genes. The method is characterised in that

a) within said time period and environment GFP and luciferase are produced:

i) GFP is produced in a, within the environment of interest, essentially known proportion to the total amount of cells of said micro-organism that are or have been alive within said chosen time period, and

ii) luciferase is present in said environment of interest in an essentially known proportion to the amount of cells alive at any moment within said chosen time period and GFP can be measured through its fluorescence and luciferase can be measured through its luminescence;

b) said micro-organism is incubated within the environment of interest and said luminescence and fluorescence is detected after said chosen time period, and

c) the growth and death rate of the said micro-organism is assessed based on the following:

i) the known proportion of luminescence to the amount of cells alive after any said chosen time period,

ii) the known proportion of fluorescence to the total amount of cells that are or have been alive within any said chosen time period.

BRIEF DESCRIPTION OF THE DRAWINGS

[0006]

Figure 1 shows plasmid pGFP+luc* including genes for both GFP and firefly luciferase.

Figure 2 shows fluorescence during growth phase of *E. coli* with plasmid pGFP+luc* at 30 °C as a function of the concentration of ethanol in the cell culture.

Figure 3 shows luminescence during growth phase of *E. coli* with plasmid pGFP+luc* at 30 °C as a function of the concentration of ethanol in the cell culture.

Figure 4 shows the amount of living cells, i.e. colony forming units, according to plating during growth phase of *E. coli* with plasmid pGFP+luc* at 30 °C as a function of the concentration of ethanol in the cell culture.

Figure 5 shows the percentage of living cells according to live/dead staining and flow cytometric analysis during growth phase of *E. coli* with plasmid pGFP+luc* at 30 °C as a function of the concentration of ethanol in the cell culture.

Figure 6 shows fluorescence before and after incubation with serum complement during growth phase of *E. coli* with plasmid pGFP+luc* at 30 °C as a function of the concentration of serum complement in the cell culture.

Figure 7 shows luminescence before and after incubation with serum complement during growth phase of *E. coli* with plasmid pGFP+luc* at 30 °C as a function of the concentration of serum complement in the cell culture.

Figure 8 shows the percentage of living cells according to plating during growth phase of *E. coli* with plasmid pGFP+luc* at 30 °C as a function of the concentration of serum complement in the cell culture.

DETAILED DESCRIPTION OF THE INVENTION

[0007] The method according to the present invention can be used to assess the growth and death rate of a micro-organism within a chosen time period in any particular environment of interest. The method is applicable if GFP and luciferase genes can be introduced to the micro-organism that code for luminescent and fluorescent products, and the products of these fulfil the following criteria:

a) luciferase luminesces in an essentially known proportion to the amount of cells of said micro-organism alive within said chosen time period; and
b) GFP fluoresces in an essentially known proportion to the amount of cells of said micro-organism that are or have been alive within said chosen time period.

[0008] In the present application the concept "micro-organism" means any micro-organism into which marker genes can be introduced so, that they will function according to the invention. "Micro-organism" can therefore stand for bacteria, yeast or fungi.

[0009] The concept of "introducing a marker gene into a micro-organism" means any method by which a marker gene can be made to function within the micro-organism according to the invention. One way of introducing marker genes into micro-organism is by constructing a recombinant strain. This can be done by transforming a strain with a plasmid including the marker genes. An alternative way to introduce reporter genes to bacteria is to utilise transposable elements. In this technique, reporter genes are inserted between insertion sequences in a delivery plasmid. The plasmid is then introduced to a cell by e.g. conjugation of transformation, and once inside the cell, genes surrounded by the insertion sequences are integrated into bacterial chromosome. Integration is stable, i.e. there is no need for a selectable marker such as antibiotic resistance.

[0010] Assessment of the growth rate and death rate of a micro-organism can be of interest in many specific environments. Within pharmaceutical research the effect of different drugs and candidates for drugs, e.g. antibiotics, on the survival of pathogenic, but also the beneficial micro-organisms of the gut, is of interest. Thus the ultimate interest is in the behaviour of these micro-organisms in a physiological environment affected by drugs.

[0011] Another vast area where the possibility of assessing growth and death rate of specific micro-organisms is of interest is that of production, processing, storage and distribution of all products for human consumption. In this area the behaviour of pathogenic or potentially harmful micro-organisms in the different environments of the life cycle of these products is of special interest and involves many different aspects such as the influence of temperature, humidity or light and the possible use of preservatives etc.

[0012] Additionally growth and death rates of micro-organisms can be of interest for environmental evaluations e.g. when evaluating the effect of emissions into the environment.

[0013] Luciferase or GFP coded by reporter genes in different embodiments of this invention can vary as long as their proportion to either the total amount of cells alive, to cells that are or have been alive, or to cells that have died

is essentially known. Growth and death rate can be assessed if cells alive, and cells that are or have been alive, can be determined.

[0014] According to one specific embodiment of the invention assessment of the growth and death rate of an *Esherichia coli* strain under the influence of different chemicals or matrixes was enabled by constructing a recombinant strain, which expresses both luciferase and GFP. Altogether the effect of a number of different chemicals and tetracyclin, as well as serum complement on said recombinant *E. coli* strain was tested and found applicable.

[0015] The invention will be described in more detail by the following study in which the growth rate and death rate of a recombinant *Esherichia coli* strain, which expresses both luciferase and GFP, is assessed under the influence of ethanol or serum complement.

Summary of the study

[0016] Genes for luciferase and green fluorescent protein have recently raised interest as reporter genes. Luciferase is an enzyme that produces luminescence in the presence of substrate luciferin, molecular oxygen and ATP. Green fluorescent protein (GFP), produces green fluorescence when excited with light. Many mutated forms of GFP have been introduced: some have different excitation and emission wavelengths from the wild type and some mutants form more stable proteins at higher temperatures.

[0017] We constructed a recombinant strain of *E. coli,* which expresses both luciferase and GFP. In our construction we used a mutant of GFP, which is more stable at temperatures over +30 °C and it matures quicker than the wild type. Luciferase was from North American firefly, *Photinus pyralis*.

[0018] The *E. coli* strain MC1061 was transformed with a plasmid including genes for both GFP and firefly luciferase. Figure 1 describes the plasmid in general. The sequence of the plasmid is disclosed in the sequence listing. Essential codings of the sequence are as follows:

lac promoter	95-199
GFP	289-1008
firefly luciferase	1044-2696
β-lactamase	3251-4111

[0019] In our construct, see Figure 1, the luciferase gene is situated next to the GFP gene and both genes are transcribed in the same direction. The transcription is started at the lac promoter in front of GFP. The lac promoter is constitutively active, because the MC1061 cells lack its repressor. The plasmid also has a gene for ampicillin resistance (β-lactamase).

[0020] The transformed *E. coli* strain was propagated under the influence of different concentrations of ethanol or serum complement.

Methods

Growth conditions

[0021] One colony from a pure culture plate was inoculated in 5 ml of LB-medium with ampicillin (100 μg/ml) and grown at +37 °C in a shaker, 250 rpm, for about three hours. After that, the number of cells per millilitre was determined with flow cytometry by using fluorescent spherical latex particles as a reference. One million cells were then removed to an erlenmeyer with 50 ml of LB medium and ampicillin. The culture was grown over night in a shaker, 190 rpm, at room temperature to prevent the culture from growing into the stationary phase during the night. In the morning, the culture was transferred to and grown in a shaker, 330 rpm, until the stationary phase was reached or used after growth at +30 °C for about 1 h to study the influence of ethanol or serum complement as described below.

Influence of chemicals on the propagation of *E. coli*

[0022] The culture obtained as described above was used to study the influence of ethanol or serum complement as follows:

Ethanol

[0023] Ethanol (Aa, Primalco Oy) was diluted into pure water to obtain concentrations of 50, 45, 25, 10, 5, 1 and 0 % of ethanol when 500 μl of said dilution was added to 500 μl of said culture in an eppendorf tube. The mixture was

vortexed and incubated for 5 minutes before measuring fluorescence and luminescence. Live cells were again counted by plating and also by live/dead staining. In the live/dead protocol used the stain *cyto* 9 stains all cells whereas propidium iodide stains only the dead cells. After staining, cells are passed through a flow cytometer, with which dead and live cells can be differentiated and their proportion determined. (Virta et al. (1998) Appl. Environ. Microbiol. 64: 515-519.)

Serum complement

[0024] The influence of serum complement on the said recombinant *E. coli* strain was studied using an incubation time of 90 min as described for a different recombinant *E. coli* strain used in Virta et al. (1998) Appl. Environ. Microbiol. 64: 515-519.

Fluorescence and luminescence measurements

[0025] The measurements were done with a combined fluoro- and luminometer, Fluoroscan Ascent FL, provided by Labsystems Ltd. (Helsinki, Finland). Cell growth was simultaneously followed with a flow cytometer.

[0026] For the measurements, 100 µl of bacterial culture was pipetted into the microtiter plate wells. Fluorescence was measured using 485 nm for excitation and 510 nm for emission. Measuring time was 20 ms. After the fluorescence measurement 100 µl of luciferin in 0.1 M citric acid-sodium citrate buffer (pH 5.0) was dispensed into the wells and the plate was shaken for two minutes (shaking diameter I mm, 1 020 rpm), after which luminescence was recorded with a measuring time of 1000 ms.

Plating

[0027] Samples for plating were diluted 10^2 to 10^7 fold with 150 mM NaCl and plated onto L agar plates (L broth containing 1.6 % agar). Colonies were counted after overnight incubation at 37 °C.

Live/dead staining and Flow cytometric analysis

[0028] Bacteria from 1 000 µl of cell culture were used for live/dead staining and flow cytometric analysis using a LIVE/DEAD BacLight bacterial viability kit (catalogue no. L-7005) for microscopy and quantitative analysis obtained from Molecular Probes Europe (Leiden, The Netherlands) and Fluoresbrite beads (diameter, 1.8 µm) obtained from Polysciences Inc. (Warrington, Pa.) as described in Virta et al. (1998) Appl. Environ. Microbiol. 64: 515-519.

Results

[0029] When the cultures were transferred to +30 °C, the cells grew logarithmically for 1—4 hours depending on the initial cell concentration. Luminescence and fluorescence rose logarithmically and were essentially constant per cell. Thus cell number could be assessed based on luminescence or fluorescence.

[0030] When ethanol was added in different concentrations to the growth medium (see Figures 4 and 5) death was, after a very short incubation period of 5 min, more or less unsignificant at ethanol concentrations below 5 % and became more significant with increasing ethanol concentration reaching very pronounced significance at ethanol concentrations above 10 %. Correspondingly fluorescence (Figure 2) was essentially constant whatever the ethanol concentration in spite of dramatically decreasing corresponding live cell count according to plate count (Figure 4) and percentage of live cells according to the live/dead staining (Figure 5) whereas luminescence (Figure 3) dropped dramatically essentially corresponding to the dramatic drop in plate count (Figure 4) and the percentage of live cells (Figure 5) with increased ethanol concentration.

[0031] The effect of serum complement on the growth and death of *E. coli* is shown in Figures 6 to 8. Fluorescence (Figure 6) and luminescence (Figure 7) are shown before (squares) and after (circles) incubation for 90 minutes with serum complement. Fluorescence (Figure 6) is slightly increased, during incubation regardless of the concentration of serum, whereas luminescence (Figure 7) decreases during incubation with increasing serum concentration. The decrease of luminescence during incubation with increasing concentrations of serum correlates clearly with the percentage of cells alive after incubation (Figure 8).

SEQUENCE LISTING

[0032]

<110> LILIUS, Esa-Matti

<120> A Method to Enable Assessment of Growth and Death of Micro-orgnaisms

<130> pGFP+luc* -plasmid

<140>
<141>

<160> 4

<170> PatentIn Ver. 2.1

<210> 1
<211> 5051
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: pGFP+luc* plasmid

<220>
<221> promoter
<222> (95)..(199)
<223> lac promoter

<220>
<221> CDS
<222> (289)..(1008)
<223> GFP

<220>
<221> CDS
<222> (1044)..(2696)
<223> firefly luciferase

<220>
<221> CDS
<222> (3251)..(4111)
<223> beta-lactamase

<400> 1

```
agcgcccaat acgcaaaccg cctctccccg cgcgttggcc gattcattaa tgcagctggc 60

acgacaggtt tcccgactgg aaagcgggca gtgagcgcaa cgcaattaat gtgagttagc 120

tcactcatta ggcaccccag gctttacact ttatgcttcc ggctcgtatg ttgtgtggaa 180

ttgtgagcgg ataacaattt cacacaggaa acagctatga ccatgattac gccaagcttg 240

catgcctgca ggtcgactct agaggatccc cgggtaccgg tcgccacc atg gtg agc  297
                                                Met Val Ser
                                                  1

aag ggc gag gag ctg ttc acc ggg gtg gtg ccc atc ctg gtc gag ctg   345
Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val Glu Leu
      5                  10                  15

gac ggc gac gta aac ggc cac aag ttc agc gtg tcc ggc gag ggc gag   393
Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu Gly Glu
 20                  25                  30                  35
```

```
ggc gat gcc acc tac ggc aag ctg acc ctg aag ttc atc tgc acc acc   441
Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys Thr Thr
                 40                  45                  50

ggc aag ctg ccc gtg ccc tgg ccc acc ctc gtg acc acc ctg acc tac   489
Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Leu Thr Tyr
             55                  60                  65

ggc gtg cag tgc ttc agc cgc tac ccc gac cac atg aag cag cac gac   537
Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Gln His Asp
         70                  75                  80

ttc ttc aag tcc gcc atg ccc gaa ggc tac gtc cag gag cgc acc atc   585
Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg Thr Ile
     85                  90                  95

ttc ttc aag gac gac ggc aac tac aag acc cgc gcc gag gtg aag ttc   633
Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val Lys Phe
100                 105                 110                 115

gag ggc gac acc ctg gtg aac cgc atc gag ctg aag ggc atc gac ttc   681
Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile Asp Phe
                120                 125                 130

aag gag gac ggc aac atc ctg ggg cac aag ctg gag tac aac tac aac   729
Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn Tyr Asn
            135                 140                 145

agc cac aac gtc tat atc atg gcc gac aag cag aag aac ggc atc aag   777
Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly Ile Lys
        150                 155                 160

gtg aac ttc aag atc cgc cac aac atc gag gac ggc agc gtg cag ctc   825
Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser Val Gln Leu
    165                 170                 175

gcc gac cac tac cag cag aac acc ccc atc ggc gac ggc ccc gtg ctg   873
Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro Val Leu
180                 185                 190                 195

ctg ccc gac aac cac tac ctg agc acc cag tcc gcc ctg agc aaa gac   921
Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser Lys Asp
                200                 205                 210

ccc aac gag aag cgc gat cac atg gtc ctg ctg gag ttc gtg acc gcc   969
Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe Val Thr Ala
            215                 220                 225

gcc ggg atc act ctc ggc atg gac gag ctg tac aag taa agcggccgct   1018
Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys
        230                 235                 240

ctagaactag tggatccccc gtacc atg gaa gac gcc aaa aac ata aag aaa   1070
                          Met Glu Asp Ala Lys Asn Ile Lys Lys
                                  245

ggc ccg gcg cca ttc tat ccg cta gag gat gga acc gct gga gag caa   1118
Gly Pro Ala Pro Phe Tyr Pro Leu Glu Asp Gly Thr Ala Gly Glu Gln
250                 255                 260                 265

ctg cat aag gct atg aag aga tac gcc ctg gtt cct gga aca att gct   1166
Leu His Lys Ala Met Lys Arg Tyr Ala Leu Val Pro Gly Thr Ile Ala
                270                 275                 280
```

```
ttt aca gat gca cat atc gag gtg aac atc acg tac gcg gaa tac ttc   1214
Phe Thr Asp Ala His Ile Glu Val Asn Ile Thr Tyr Ala Glu Tyr Phe
            285                 290                 295

gaa atg tcc gtt cgg ttg gca gaa gct atg aaa cga tat ggg ctg aat   1262
Glu Met Ser Val Arg Leu Ala Glu Ala Met Lys Arg Tyr Gly Leu Asn
        300                 305                 310

aca aat cac aga atc gtc gta tgc agt gaa aac tct ctt caa ttc ttt   1310
Thr Asn His Arg Ile Val Val Cys Ser Glu Asn Ser Leu Gln Phe Phe
    315                 320                 325

atg ccg gtg ttg ggc gcg tta ttt atc gga gtt gca gtt gcg ccc gcg   1358
Met Pro Val Leu Gly Ala Leu Phe Ile Gly Val Ala Val Ala Pro Ala
330                 335                 340                 345

aac gac att tat aat gaa cgt gaa ttg ctc aac agt atg aac att tcg   1406
Asn Asp Ile Tyr Asn Glu Arg Glu Leu Leu Asn Ser Met Asn Ile Ser
                350                 355                 360

cag cct acc gta gtg ttt gtt tcc aaa aag ggg ttg caa aaa att ttg   1454
Gln Pro Thr Val Val Phe Val Ser Lys Lys Gly Leu Gln Lys Ile Leu
            365                 370                 375

aac gtg caa aaa aaa tta cca ata atc cag aaa att att atc atg gat   1502
Asn Val Gln Lys Lys Leu Pro Ile Ile Gln Lys Ile Ile Ile Met Asp
        380                 385                 390

tct aaa acg gat tac cag gga ttt cag tcg atg tac acg ttc gtc aca   1550
Ser Lys Thr Asp Tyr Gln Gly Phe Gln Ser Met Tyr Thr Phe Val Thr
    395                 400                 405

tct cat cta cct ccc ggt ttt aat gaa tac gat ttt gta cca gag tcc   1598
Ser His Leu Pro Pro Gly Phe Asn Glu Tyr Asp Phe Val Pro Glu Ser
410                 415                 420                 425

ttt gat cgt gac aaa aca att gca ctg ata atg aac tcc tct gga tct   1646
Phe Asp Arg Asp Lys Thr Ile Ala Leu Ile Met Asn Ser Ser Gly Ser
                430                 435                 440

act ggg tta cct aag ggt gtg gcc ctt ccg cat aga act gcc tgc gtc   1694
Thr Gly Leu Pro Lys Gly Val Ala Leu Pro His Arg Thr Ala Cys Val
            445                 450                 455

aga ttc tcg cat gcc aga gat cct att ttt ggc aat caa atc att ccg   1742
Arg Phe Ser His Ala Arg Asp Pro Ile Phe Gly Asn Gln Ile Ile Pro
        460                 465                 470

gat act gcg att tta agt gtt gtt cca ttc cat cac ggt ttt gga atg   1790
Asp Thr Ala Ile Leu Ser Val Val Pro Phe His His Gly Phe Gly Met
    475                 480                 485

ttt act aca ctc gga tat ttg ata tgt gga ttt cga gtc gtc tta atg   1838
Phe Thr Thr Leu Gly Tyr Leu Ile Cys Gly Phe Arg Val Val Leu Met
490                 495                 500                 505

tat aga ttt gaa gaa gag ctg ttt tta cga tcc ctt cag gat tac aaa   1886
Tyr Arg Phe Glu Glu Glu Leu Phe Leu Arg Ser Leu Gln Asp Tyr Lys
                510                 515                 520

att caa agt gcg ttg cta gta cca acc cta ttt tca ttc ttc gcc aaa   1934
Ile Gln Ser Ala Leu Leu Val Pro Thr Leu Phe Ser Phe Phe Ala Lys
            525                 530                 535
```

```
agc act ctg att gac aaa tac gat tta tct aat tta cac gaa att gct   1982
Ser Thr Leu Ile Asp Lys Tyr Asp Leu Ser Asn Leu His Glu Ile Ala
        540                 545                 550

tct ggg ggc gca cct ctt tcg aaa gaa gtc ggg gaa gcg gtt gca aaa   2030
Ser Gly Gly Ala Pro Leu Ser Lys Glu Val Gly Glu Ala Val Ala Lys
    555                 560                 565

cgc ttc cat ctt cca ggg ata cga caa gga tat ggg ctc act gag act   2078
Arg Phe His Leu Pro Gly Ile Arg Gln Gly Tyr Gly Leu Thr Glu Thr
570                 575                 580                 585

aca tca gct att ctg att aca ccc gag ggg gat gat aaa ccg ggc gcg   2126
Thr Ser Ala Ile Leu Ile Thr Pro Glu Gly Asp Asp Lys Pro Gly Ala
                590                 595                 600

gtc ggt aaa gtt gtt cca ttt ttt gaa gcg aag gtt gtg gat ctg gat   2174
Val Gly Lys Val Val Pro Phe Phe Glu Ala Lys Val Val Asp Leu Asp
            605                 610                 615

acc ggg aaa acg ctg ggc gtt aat cag aga ggc gaa tta tgt gtc aga   2222
Thr Gly Lys Thr Leu Gly Val Asn Gln Arg Gly Glu Leu Cys Val Arg
        620                 625                 630

gga cct atg att atg tcc ggt tat gta aac aat ccg gaa gcg acc aac   2270
Gly Pro Met Ile Met Ser Gly Tyr Val Asn Asn Pro Glu Ala Thr Asn
    635                 640                 645

gcc ttg att gac aag gat gga tgg cta cat tct gga gac ata gct tac   2318
Ala Leu Ile Asp Lys Asp Gly Trp Leu His Ser Gly Asp Ile Ala Tyr
650                 655                 660                 665

tgg gac gaa gac gaa cac ttc ttc ata gtt gac cgc ttg aag tct tta   2366
Trp Asp Glu Asp Glu His Phe Phe Ile Val Asp Arg Leu Lys Ser Leu
                670                 675                 680

att aaa tac aaa gga tac cag gtg gcc ccc gct gaa ttg gag tcg ata   2414
Ile Lys Tyr Lys Gly Tyr Gln Val Ala Pro Ala Glu Leu Glu Ser Ile
            685                 690                 695

ttg tta caa cac ccc aac atc ttc gac gcg ggc gtg gca ggt ctt ccc   2462
Leu Leu Gln His Pro Asn Ile Phe Asp Ala Gly Val Ala Gly Leu Pro
        700                 705                 710

gac gat gac gcc ggt gaa ctt ccc gcc gcc gtt gtt gtt ttg gag cac   2510
Asp Asp Asp Ala Gly Glu Leu Pro Ala Ala Val Val Val Leu Glu His
    715                 720                 725

gga aag acg atg acg gaa aaa gag atc gtg gat tac gtc gcc agt caa   2558
Gly Lys Thr Met Thr Glu Lys Glu Ile Val Asp Tyr Val Ala Ser Gln
730                 735                 740                 745

gta aca acc gcc aaa aag ttg cgc gga gga gtt gtg ttt gtg gac gaa   2606
Val Thr Thr Ala Lys Lys Leu Arg Gly Gly Val Val Phe Val Asp Glu
                750                 755                 760

gta ccg aaa ggt ctt acc gga aaa ctc gac gca aga aaa atc aga gag   2654
Val Pro Lys Gly Leu Thr Gly Lys Leu Asp Ala Arg Lys Ile Arg Glu
            765                 770                 775

atc ctc ata aag gcc aag aag ggc gga aag tcc aaa ttg taa           2696
Ile Leu Ile Lys Ala Lys Lys Gly Gly Lys Ser Lys Leu
        780                 785                 790
```

```
aatgtaactg tattcagcga tgacgaaatt cttagctatt gtaatactct aggggctgca 2756

ggaattcgat atcaagctta tcgataccgt cgacctcgag gggggccct ttcgtctcgc 2816

gcgtttcggt gatgacggtg aaaacctctg acacatgcag ctcccggaga cggtcacagc 2876

ttgtctgtaa gcggatgccg ggagcagaca agcccgtcag ggcgcgtcag cgggtgttgg 2936

cgggtgtcgg ggctggctta actatgcggc atcagagcag attgtactga gagtgcacca 2996

tatgcggtgt gaaataccgc acagatgcgt aaggagaaaa taccgcatca ggcggcctta 3056

agggcctcgt gatacgccta tttttatagg ttaatgtcat gataataatg gtttcttaga 3116

cgtcaggtgg cacttttcgg ggaaatgtgc gcggaacccc tatttgttta tttttctaaa 3176

tacattcaaa tatgtatccg ctcatgagac aataaccctg ataaatgctt caataatatt 3236

gaaaaaggaa gagt atg agt att caa cat ttc cgt gtc gcc ctt att ccc   3286
                Met Ser Ile Gln His Phe Arg Val Ala Leu Ile Pro
                    795                 800

ttt ttt gcg gca ttt tgc ctt cct gtt ttt gct cac cca gaa acg ctg   3334
Phe Phe Ala Ala Phe Cys Leu Pro Val Phe Ala His Pro Glu Thr Leu
    805                 810                 815

gtg aaa gta aaa gat gct gaa gat cag ttg ggt gca cga gtg ggt tac   3382
Val Lys Val Lys Asp Ala Glu Asp Gln Leu Gly Ala Arg Val Gly Tyr
820                 825                 830                 835

atc gaa ctg gat ctc aac agc ggt aag atc ctt gag agt ttt cgc ccc   3430
Ile Glu Leu Asp Leu Asn Ser Gly Lys Ile Leu Glu Ser Phe Arg Pro
                840                 845                 850

gaa gaa cgt ttt cca atg atg agc act ttt aaa gtt ctg cta tgt ggc   3478
Glu Glu Arg Phe Pro Met Met Ser Thr Phe Lys Val Leu Leu Cys Gly
            855                 860                 865

gcg gta tta tcc cgt att gac gcc ggg caa gag caa ctc ggt cgc cgc   3526
Ala Val Leu Ser Arg Ile Asp Ala Gly Gln Glu Gln Leu Gly Arg Arg
        870                 875                 880

ata cac tat tct cag aat gac ttg gtt gag tac tca cca gtc aca gaa   3574
Ile His Tyr Ser Gln Asn Asp Leu Val Glu Tyr Ser Pro Val Thr Glu
    885                 890                 895

aag cat ctt acg gat ggc atg aca gta aga gaa tta tgc agt gct gcc   3622
Lys His Leu Thr Asp Gly Met Thr Val Arg Glu Leu Cys Ser Ala Ala
900                 905                 910                 915

ata acc atg agt gat aac act gcg gcc aac tta ctt ctg aca acg atc   3670
Ile Thr Met Ser Asp Asn Thr Ala Ala Asn Leu Leu Leu Thr Thr Ile
                920                 925                 930

gga gga ccg aag gag cta acc gct ttt ttg cac aac atg ggg gat cat   3718
Gly Gly Pro Lys Glu Leu Thr Ala Phe Leu His Asn Met Gly Asp His
            935                 940                 945

gta act cgc ctt gat cgt tgg gaa ccg gag ctg aat gaa gcc ata cca   3766
Val Thr Arg Leu Asp Arg Trp Glu Pro Glu Leu Asn Glu Ala Ile Pro
        950                 955                 960

aac gac gag cgt gac acc acg atg cct gta gca atg gca aca acg ttg   3814
Asn Asp Glu Arg Asp Thr Thr Met Pro Val Ala Met Ala Thr Thr Leu
    965                 970                 975
```

```
cgc aaa cta tta act ggc gaa cta ctt act cta gct tcc cgg caa caa   3862
Arg Lys Leu Leu Thr Gly Glu Leu Leu Thr Leu Ala Ser Arg Gln Gln
980                 985                 990                 995

tta ata gac tgg atg gag gcg gat aaa gtt gca gga cca ctt ctg cgc   3910
Leu Ile Asp Trp Met Glu Ala Asp Lys Val Ala Gly Pro Leu Leu Arg
                1000                1005                1010

tcg gcc ctt ccg gct ggc tgg ttt att gct gat aaa tct gga gcc ggt   3958
Ser Ala Leu Pro Ala Gly Trp Phe Ile Ala Asp Lys Ser Gly Ala Gly
            1015                1020                1025

gag cgt ggg tct cgc ggt atc att gca gca ctg ggg cca gat ggt aag   4006
Glu Arg Gly Ser Arg Gly Ile Ile Ala Ala Leu Gly Pro Asp Gly Lys
        1030                1035                1040

ccc tcc cgt atc gta gtt atc tac acg acg ggg agt cag gca act atg   4054
Pro Ser Arg Ile Val Val Ile Tyr Thr Thr Gly Ser Gln Ala Thr Met
    1045                1050                1055

gat gaa cga aat aga cag atc gct gag ata ggt gcc tca ctg att aag   4102
Asp Glu Arg Asn Arg Gln Ile Ala Glu Ile Gly Ala Ser Leu Ile Lys
1060                1065                1070                1075

cat tgg taa ctgtcagacc aagtttactc atatatactt tagattgatt           4151
His Trp

taaaacttca tttttaattt aaaaggatct aggtgaagat ccttttttgat aatctcatga 4211

ccaaaatccc ttaacgtgag ttttcgttcc actgagcgtc agaccccgta gaaaagatca 4271

aaggatcttc ttgagatcct ttttttctgc gcgtaatctg ctgcttgcaa acaaaaaaac 4331

caccgctacc agcggtggtt tgtttgccgg atcaagagct accaactctt tttccgaagg 4391

taactggctt cagcagagcg cagataccaa atactgtcct tctagtgtag ccgtagttag 4451

gccaccactt caagaactct gtagcaccgc ctacatacct cgctctgcta atcctgttac 4511

cagtggctgc tgccagtggc gataagtcgt gtcttaccgg gttggactca agacgatagt 4571

taccggataa ggcgcagcgg tcgggctgaa cgggggggttc gtgcacacag cccagcttgg 4631

agcgaacgac ctacaccgaa ctgagatacc tacagcgtga gctatgagaa agcgccacgc 4691

ttcccgaagg gagaaaggcg gacaggtatc cggtaagcgg cagggtcgga acaggagagc 4751

gcacgaggga gcttccaggg ggaaacgcct ggtatcttta tagtcctgtc gggtttcgcc 4811

acctctgact tgagcgtcga tttttgtgat gctcgtcagg ggggcggagc ctatggaaaa 4871

acgccagcaa cgcggccttt ttacggttcc tggccttttg ctggcctttt gctcacatgt 4931

tctttcctgc gttatcccct gattctgtgg ataaccgtat taccgccttt gagtgagctg 4991

ataccgctcg ccgcagccga acgaccgagc gcagcgagtc agtgagcgag gaagcggaag 5051
```

<210> 2
<211> 239
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence: pGFP+luc* plasmid

<400> 2

```
Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
 1               5                  10                  15
Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
            20                  25                  30
Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
        35                  40                  45
Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
    50                  55                  60
Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
65                  70                  75                  80
Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
                85                  90                  95
Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
            100                 105                 110
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
        115                 120                 125
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
    130                 135                 140
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
145                 150                 155                 160
Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
                165                 170                 175
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
            180                 185                 190
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
        195                 200                 205
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
    210                 215                 220
Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys
225                 230                 235
```

<210> 3

<211> 550

<212> PRT

<213> Artificial Sequence

<223> Description of Artificial Sequence: pGFP+luc* plasmid

<400> 3

```
Met Glu Asp Ala Lys Asn Ile Lys Lys Gly Pro Ala Pro Phe Tyr Pro
  1               5                  10                  15
Leu Glu Asp Gly Thr Ala Gly Glu Gln Leu His Lys Ala Met Lys Arg
            20                  25                  30
Tyr Ala Leu Val Pro Gly Thr Ile Ala Phe Thr Asp Ala His Ile Glu
        35                  40                  45
Val Asn Ile Thr Tyr Ala Glu Tyr Phe Glu Met Ser Val Arg Leu Ala
    50                  55                  60
Glu Ala Met Lys Arg Tyr Gly Leu Asn Thr Asn His Arg Ile Val Val
65                  70                  75                  80
Cys Ser Glu Asn Ser Leu Gln Phe Phe Met Pro Val Leu Gly Ala Leu
                85                  90                  95
Phe Ile Gly Val Ala Val Ala Pro Ala Asn Asp Ile Tyr Asn Glu Arg
            100                 105                 110
Glu Leu Leu Asn Ser Met Asn Ile Ser Gln Pro Thr Val Val Phe Val
        115                 120                 125
Ser Lys Lys Gly Leu Gln Lys Ile Leu Asn Val Gln Lys Lys Leu Pro
    130                 135                 140
Ile Ile Gln Lys Ile Ile Ile Met Asp Ser Lys Thr Asp Tyr Gln Gly
145                 150                 155                 160
Phe Gln Ser Met Tyr Thr Phe Val Thr Ser His Leu Pro Pro Gly Phe
                165                 170                 175
Asn Glu Tyr Asp Phe Val Pro Glu Ser Phe Asp Arg Asp Lys Thr Ile
            180                 185                 190
```

```
Ala Leu Ile Met Asn Ser Ser Gly Ser Thr Gly Leu Pro Lys Gly Val
        195                 200                 205
Ala Leu Pro His Arg Thr Ala Cys Val Arg Phe Ser His Ala Arg Asp
    210                 215                 220
Pro Ile Phe Gly Asn Gln Ile Ile Pro Asp Thr Ala Ile Leu Ser Val
225                 230                 235                 240
Val Pro Phe His His Gly Phe Gly Met Phe Thr Thr Leu Gly Tyr Leu
                245                 250                 255
Ile Cys Gly Phe Arg Val Val Leu Met Tyr Arg Phe Glu Glu Glu Leu
            260                 265                 270
Phe Leu Arg Ser Leu Gln Asp Tyr Lys Ile Gln Ser Ala Leu Leu Val
        275                 280                 285
Pro Thr Leu Phe Ser Phe Phe Ala Lys Ser Thr Leu Ile Asp Lys Tyr
    290                 295                 300
Asp Leu Ser Asn Leu His Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser
305                 310                 315                 320
Lys Glu Val Gly Glu Ala Val Ala Lys Arg Phe His Leu Pro Gly Ile
                325                 330                 335
Arg Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Ile Leu Ile Thr
            340                 345                 350
Pro Glu Gly Asp Asp Lys Pro Gly Ala Val Gly Lys Val Val Pro Phe
        355                 360                 365
Phe Glu Ala Lys Val Val Asp Leu Asp Thr Gly Lys Thr Leu Gly Val
    370                 375                 380
Asn Gln Arg Gly Glu Leu Cys Val Arg Gly Pro Met Ile Met Ser Gly
385                 390                 395                 400
Tyr Val Asn Asn Pro Glu Ala Thr Asn Ala Leu Ile Asp Lys Asp Gly
                405                 410                 415
Trp Leu His Ser Gly Asp Ile Ala Tyr Trp Asp Glu Asp Glu His Phe
            420                 425                 430
Phe Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln
        435                 440                 445
Val Ala Pro Ala Glu Leu Glu Ser Ile Leu Leu Gln His Pro Asn Ile
    450                 455                 460
Phe Asp Ala Gly Val Ala Gly Leu Pro Asp Asp Asp Ala Gly Glu Leu
465                 470                 475                 480
Pro Ala Ala Val Val Val Leu Glu His Gly Lys Thr Met Thr Glu Lys
                485                 490                 495
Glu Ile Val Asp Tyr Val Ala Ser Gln Val Thr Thr Ala Lys Lys Leu
            500                 505                 510
Arg Gly Gly Val Val Phe Val Asp Glu Val Pro Lys Gly Leu Thr Gly
        515                 520                 525
Lys Leu Asp Ala Arg Lys Ile Arg Glu Ile Leu Ile Lys Ala Lys Lys
    530                 535                 540
Gly Gly Lys Ser Lys Leu
545                 550
```

<210> 4
<211> 286
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence: pGFP+luc* plasmid

<400> 4

```
Met Ser Ile Gln His Phe Arg Val Ala Leu Ile Pro Phe Phe Ala Ala
 1               5                  10                  15
Phe Cys Leu Pro Val Phe Ala His Pro Glu Thr Leu Val Lys Val Lys
            20                  25                  30
Asp Ala Glu Asp Gln Leu Gly Ala Arg Val Gly Tyr Ile Glu Leu Asp
        35                  40                  45
Leu Asn Ser Gly Lys Ile Leu Glu Ser Phe Arg Pro Glu Glu Arg Phe
    50                  55                  60
Pro Met Met Ser Thr Phe Lys Val Leu Leu Cys Gly Ala Val Leu Ser
65                  70                  75                  80
```

```
Arg Ile Asp Ala Gly Gln Glu Gln Leu Gly Arg Arg Ile His Tyr Ser
                85                  90                  95
Gln Asn Asp Leu Val Glu Tyr Ser Pro Val Thr Glu Lys His Leu Thr
            100                 105                 110
Asp Gly Met Thr Val Arg Glu Leu Cys Ser Ala Ala Ile Thr Met Ser
        115                 120                 125
Asp Asn Thr Ala Ala Asn Leu Leu Leu Thr Thr Ile Gly Gly Pro Lys
    130                 135                 140
Glu Leu Thr Ala Phe Leu His Asn Met Gly Asp His Val Thr Arg Leu
145                 150                 155                 160
Asp Arg Trp Glu Pro Glu Leu Asn Glu Ala Ile Pro Asn Asp Glu Arg
                165                 170                 175
Asp Thr Thr Met Pro Val Ala Met Ala Thr Thr Leu Arg Lys Leu Leu
            180                 185                 190
Thr Gly Glu Leu Leu Thr Leu Ala Ser Arg Gln Gln Leu Ile Asp Trp
        195                 200                 205
Met Glu Ala Asp Lys Val Ala Gly Pro Leu Leu Arg Ser Ala Leu Pro
    210                 215                 220
Ala Gly Trp Phe Ile Ala Asp Lys Ser Gly Ala Gly Glu Arg Gly Ser
225                 230                 235                 240
Arg Gly Ile Ile Ala Ala Leu Gly Pro Asp Gly Lys Pro Ser Arg Ile
                245                 250                 255
Val Val Ile Tyr Thr Thr Gly Ser Gln Ala Thr Met Asp Glu Arg Asn
            260                 265                 270
Arg Gln Ile Ala Glu Ile Gly Ala Ser Leu Ile Lys His Trp
        275                 280                 285
```

Claims

1. A method to enable the assessment of the growth rate and death rate of a micro-organism within a chosen time period in an environment of interest by introducing into said micro-organism a green fluorescent protein (GFP) and a luciferase reporter gene, which method is **characterised in that**

   a) within said tune period and environment GFP and luciferase are produced:

      i) GFP is produced in a, within the environment of interest, essentially known proportion to the total amount of cells of said micro-organism that are or have been alive within said chosen time period, and

      ii) luciferase is present in said environment of interest in an essentially known proportion to the amount of cells alive at any moment within said chosen time period,

   and GFP can be measured through its fluorescence and luciferase can be measured through its luminescence;

   b) said micro-organism is incubated within the environment of interest and said luminescence and fluorescence is detected after said chosen time period, and

c) the growth and death rate of the said micro-organism is assessed based on the following:

i) the known proportion of luminescence to the amount of cells alive after any said chosen time period, and

ii) the known proportion of fluorescence to the total amount of cells that are or have been alive within any said chosen time period.

2. The method according to claim 1 **characterised in that** said micro-organism is a gram negative bacteria, e.g. *Escherichia coli.*

3. The method according to claim 1 or 2 **characterised in that** said reporter genes are introduced into said micro-organism in a plasmid.

4. A method according to the method of claim 3 **characterised in that** said plasmid is pGFP+luc* having SEQ ID NO: 1.

Patentansprüche

1. Verfahren zur Ermöglichung der Bewertung der Wachstumsrate und der Todesrate eines Mikroorganismus innerhalb eines gewählten Zeitraums in einer Umgebung von Interesse durch Einführung eines grünfluoreszierenden Proteins (GFP) und eines Luziferasereportergens in den Mikroorganismus, wobei das Verfahren dadurch charakterisiert ist, dass

a) innerhalb des Zeitraums und in der Umgebung GFP und Luziferase produziert werden:

(i) GFP wird in der Umgebung von Interesse in einem im Wesentlichen bekannten Verhältnis zu der Gesamtmenge an Zellen des Mikroorganismus, die in diesem gewählten Zeitraum lebendig sind oder lebendig waren, produziert und
(ii) Luziferase liegt in der Umgebung von Interesse in einem im Wesentlichen bekannten Verhältnis zu der Menge an Zellen, die zu einem beliebigen Zeitpunkt innerhalb dieses gewählten Zeitraums lebendig sind, vor, und GFP kann durch seine Fluoreszenz gemessen werden und Luziferase kann durch ihre Lumineszenz gemessen werden;

b) der Mikroorganismus in der Umgebung von Interesse inkubiert wird und die Lumineszenz und Fluoreszenz nach dem gewählten Zeitraum detektiert wird und

c) die Wachstums- und Todesrate des Mikroorganismus auf der Basis des Folgenden bewertet werden:

(i) dem bekannten Verhältnis der Lumineszenz zu der Menge an Zellen, die nach dem gewählten Zeitraum lebendig sind, und

(ii) dem bekannten Verhältnis der Fluoreszenz zu der Gesamtmenge an Zellen, die innerhalb des gewählten Zeitraums lebendig sind oder lebendig waren.

2. Verfahren nach Anspruch 1, **dadurch gekennzeichnet, dass** der Mikroorganismus ein Gram-negatives Bakterium, z.B. *Escherichia coli,* ist.

3. Verfahren nach Anspruch 1 oder 2, **dadurch gekennzeichnet, dass** die Reportergene in einem Plasmid in den Mikroorganismus eingeführt werden.

4. Verfahren nach Anspruch 3, **dadurch gekennzeichnet, dass** das Plasmid pGFP+luc* ist, das SEQ ID NO: 1 hat.

Revendications

1. Procédé pour permettre l'estimation du taux de croissance et du taux de disparition d'un micro-organisme dans un laps de temps choisi dans un environnement concerné en introduisant dans ledit micro-organisme une protéine

fluorescente verte (GFP) et un gène rapporteur de luciférase, lequel procédé est **caractérisé en ce que**

a) dans ledit laps de temps et dans ledit environnement une protéine GFP et de la luciférase sont produites :

i) La protéine GFP est produite, dans l'environnement concerné, essentiellement dans une proportion connue par rapport à la quantité totale de cellules du micro-organisme qui sont ou ont été vivantes dans ledit laps de temps choisi, et
ii) la luciférase est présente dans ledit environnement concerné dans une proportion essentiellement connue par rapport à la quantité de cellules vivantes à tout moment dans ledit laps de temps choisi,

et la GFP peut être mesurée au moyen de sa fluorescence et la luciférase peut être mesurée au moyen de sa luminescence ;
b) ledit micro-organisme est incubé dans l'environnement concerné et ladite luminescence et ladite fluorescence sont détectées après ledit laps de temps choisi, et
c) les taux de croissance et de disparition dudit micro-organisme sont estimés sur la base de ce qui suit :

i) la proportion connue de luminescence par rapport à la quantité de cellules vivantes après un quelconque laps de temps choisi, et
ii) la proportion connue de fluorescence par rapport à la quantité totale de cellules qui sont ou ont été vivantes dans ledit laps de temps quelconque choisi.

2. Procédé selon la revendication 1 **caractérisé en ce que** ledit micro-organisme est une bactérie à gram négatif, par exemple une Escherichia Coli.

3. Procédé selon la revendication 1 ou 2, **caractérisé en ce que** lesdits gènes rapporteurs sont introduits dans ledit micro-organisme dans un plasmide.

4. Procédé selon le procédé de la revendication 3 **caractérisé en ce que** ledit plasmide est pGFP+luc* comportant la séquence SEQ ID NO : 1.

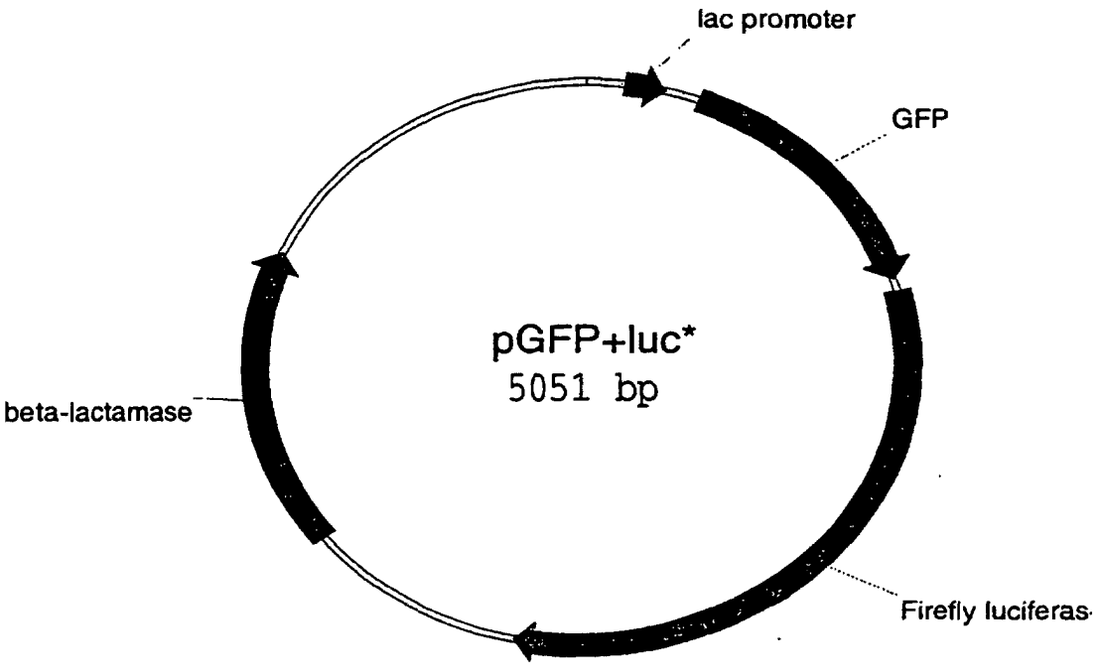

lac promoter
GFP
pGFP+luc*
5051 bp
beta-lactamase
Firefly luciferas

Figure 1

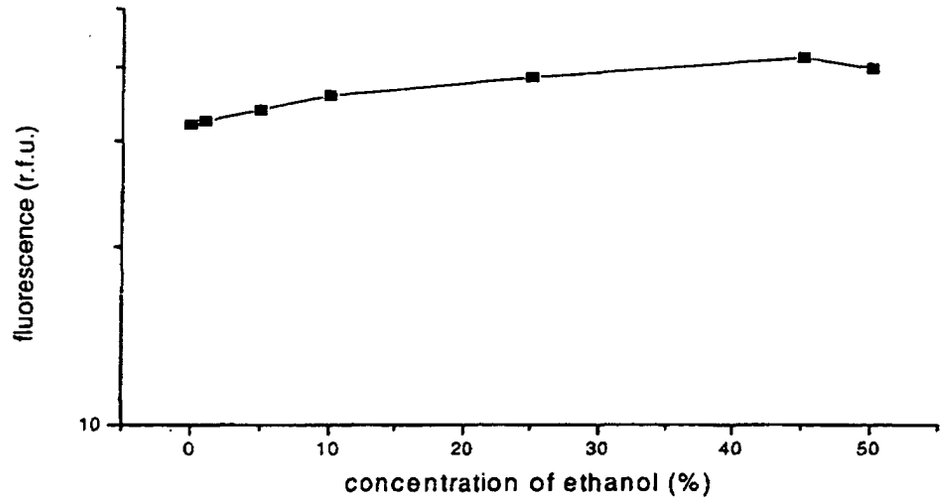


Figure 2

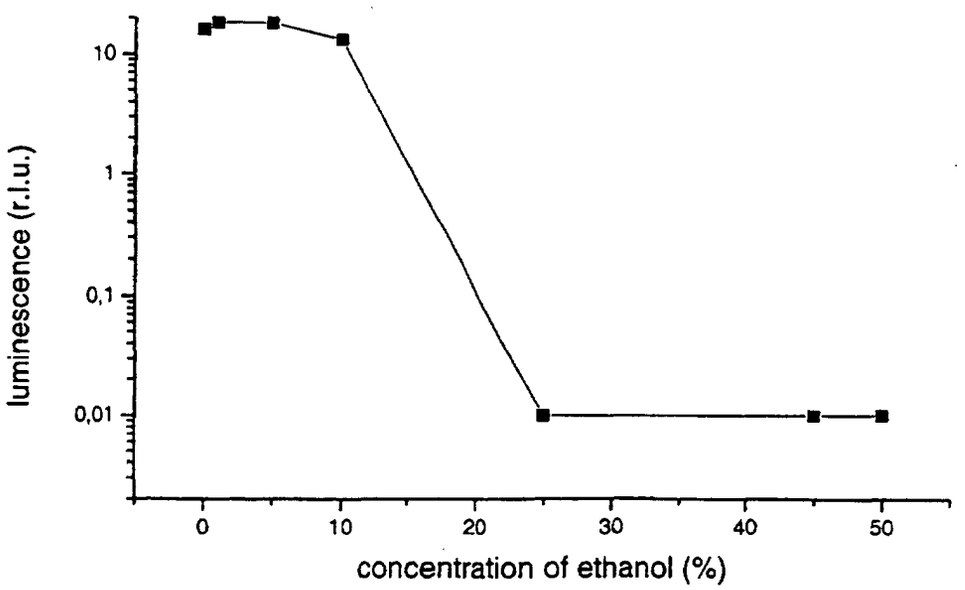


Figure 3

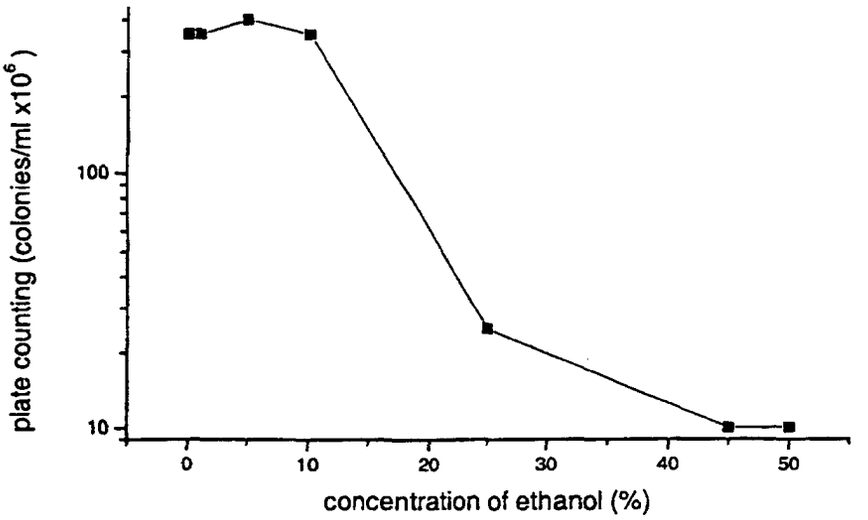


Figure 4

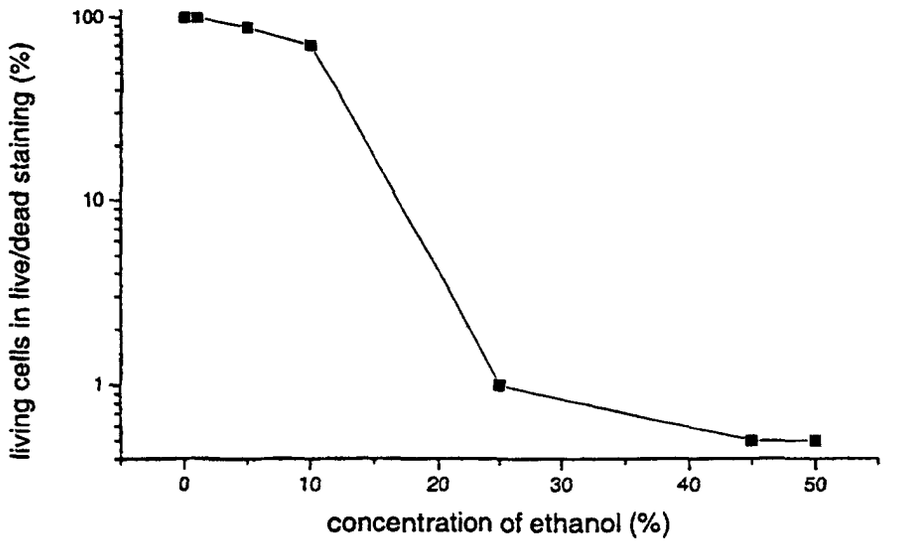


Figure 5

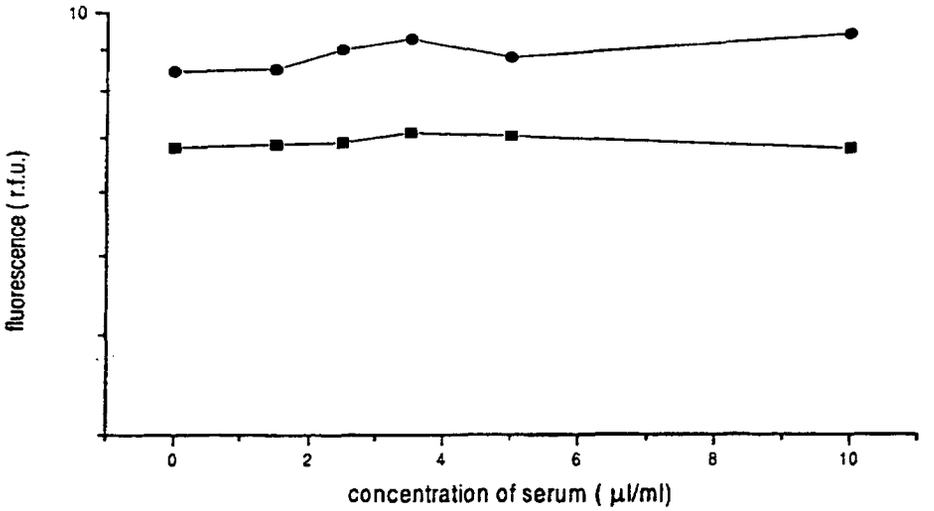


Figure 6

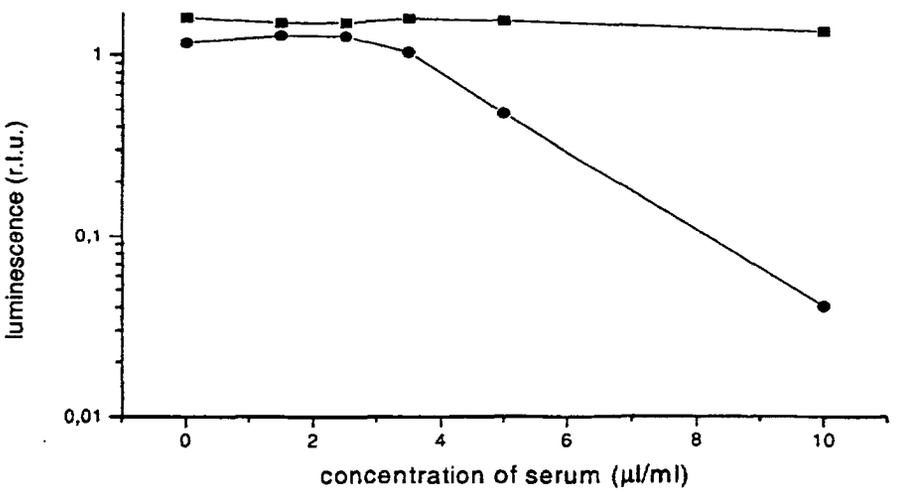


Figure 7

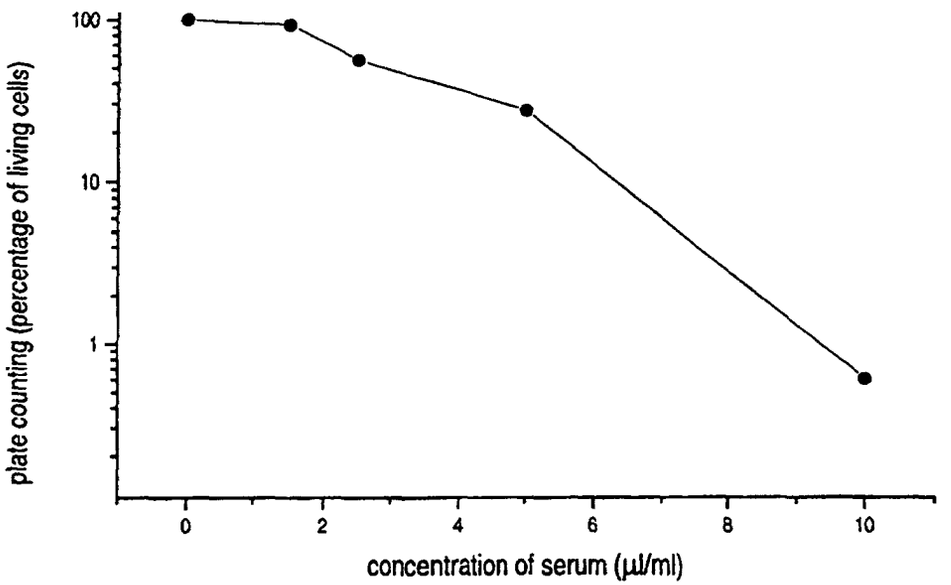

100
10
1
plate counting (percentage of living cells)
0
2
4
6
8
10
concentration of serum (µl/ml)

Figure 8